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Lysosomal alterations induced in cultured rat fibroblasts by long-term exposure to low concentrations of azithromycin

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Computer-aided simulations suggest that the doses and schedules of administration of azithromycin proposed in treatment and prophylaxis of *Mycobacterium avium* complex (MAC) in AIDS patients will result in drug concentrations in serum and extracellular fluids remaining for sustained periods of time in the 0.03–0.1 mg/L range. We exposed cultured rat embryo fibroblasts to these concentrations (and multiples up to 20 mg/L) for up to 16 days. Electron microscopy showed that after 7 days' incubation in 0.03 mg/L azithromycin, there was conspicuous accumulation of osmiophilic, lamellar structures (myeloid bodies) in lysosomes, suggesting the onset of a phospholipidosis. Assay of total cell phospholipids and cholesterol showed significant increases in cells exposed to ≥1 to 5 mg/L of azithromycin in association with hyperactivity of the lysosomal enzyme cathepsin B. The data suggest that azithromycin, at extracellular concentrations pertinent to its use for MAC treatment, and perhaps also prophylaxis, causes limited morphological alterations of the lysosomes in cultured cells which are of the same nature as those developing rapidly and extensively at higher concentrations.

Introduction

Azithromycin, the first clinically developed azalide,[1,2] is characterized by an exceptionally high accumulation in tissues.[3] In cultured cells, it localizes mainly in lysosomes,[4–6] where it is concentrated up to 1000-fold. This very high concentration is associated with the rapid development of typical alterations of the structure and function of lysosomes when the extracellular concentration is set at 10 mg/L.[7,8] However, little is known about the potential effects of long-term exposure of cells to lower, more clinically relevant concentrations. Such studies have been considered of little interest since azithromycin was originally proposed specifically for short courses of treatment (3–5 days). The recent extension of the indications for azithromycin to the long-term prophylaxis and the treatment of *Mycobacterium avium* complex (MAC) infections in AIDS patients has prompted us to re-examine its safety in relation to lysosomes, especially since pre-clinical published data are scanty in this area.[9,10] The present study used a model designed to mimic and assess the situation prevailing *in vivo* during the prophylactic (1200 mg once-a-week[11]) and therapeutic (500 mg once-a-day[12]) uses of azithromycin in MAC infections.

Materials and methods

Computer modelling of serum and cellular pharmacokinetics

Pharmacokinetic profiles of free azithromycin in the central compartment (plasma and extracellular fluids) for regimens of 1200 mg orally once-a-week for 3 weeks (prophylactic treatment) and of 500 mg orally once daily for 15 days (therapeutic treatment) were obtained by a mathematical model developed earlier.[13] Calculations were based on published data on protein binding of the drug,[3] and on serum pharmacokinetic parameters of 500 mg azithromycin given orally[14] and 1200 mg azithromycin

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(personal communication, G. Foulds, Pfizer Inc., Groton, CT, USA). These profiles were then used to predict the corresponding cellular concentrations, assuming that the cellular content of azithromycin at equilibrium is directly proportional to its extracellular concentration. This has been proven correct for short-term experiments.[5] The mathematical aspects of our procedures are given in the Appendix. In all calculations, we have assumed that the extracellular concentration is not influenced by the accumulation of the drug in cells, which is an almost acceptable approximation *in vitro* since the amount of drug stored in cells under our experimental conditions (see below) is <10% of the total amount of available drug, and is also true *in vivo* since the accumulation of the drug in tissues is already taken into account by the overall plasma pharmacokinetic model.

Materials

Unlabelled azithromycin (Pfizer s.a., Brussels, Belgium) was dissolved in dimethylsulphoxide or in 0.1 N HCl, and thereafter diluted to appropriate concentrations in the culture medium. A fresh stock solution was made for each of the two parts of the experiment. Radiolabelled azithromycin, in ethanolic solution, was supplied as the *N*-[methyl-^{14}C] compound (Pfizer Inc.) at a specific radioactivity of 53.07 mCi/mmol. Pregnant Wistar rats for preparing fibroblasts were supplied by the Proefdieren Centrum (KUL, Louvain, Belgium). All culture media were supplied by Gibco BRL (Paisley, UK) and other reagents by E. Merck AG (Darmstadt, Germany) or Sigma Chemical Co. (St Louis, MO, USA).

Cell culture and treatment

Primary cultures of rat fibroblasts were obtained by trypsinization of 18 day-old embryo carcasses.[15] Cells were grown to confluency in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum in a 5% CO_2 atmosphere for 3 days, after which they were incubated for 7 or 8 days with azithromycin (except for controls) in the same medium. Half of the dishes were then harvested, and cells from the other half were trypsinized and subcultured for an additional period of 7–8 days in fresh medium containing the same concentration of drug, again except in controls. In the experimental conditions used, the extracellular concentration of azithromycin remained almost stable during all the incubation period (only approximately 6% of the initial extracellular content was lost after 7 days).

Electron microscopy

Cells were fixed *in situ* with 1% glutaraldehyde in PBS, post-fixed with reduced OsO_4, stained *en bloc* with 1% uranyl acetate, and dehydrated in ethanol. Observations were made at 80 kV on 65 nm sections contrasted by uranyl acetate and lead citrate in a Philips CM-12 electron microscope.

Biochemical and microbiological assays

Cells were harvested by scraping after four gentle rinses in ice-cold PBS and suspended in distilled water with a brief sonication to achieve complete disruption. For cells incubated with 0.03–1 mg/L of azithromycin, the drug content of cells was determined by radioassay, the cells having been cultured in the presence of ^{14}C-labelled azithromycin. For cells incubated with 1–20 mg/L of azithromycin, we used a microbiological assay with *Bacillus subtilis* as test organism (lower detection limit: 1 mg/L).[16] To check the comparability of the results obtained by these two methods, cells incubated with 1 mg/L of ^{14}C-labelled azithromycin for 14 days were assayed for their azithromycin content by both techniques; similar values were obtained with the two methods: 0.703 ± 0.006 (SD) μg/mg protein for the radioassay and 0.7 ± 0.1 μg/mg protein for the microbiological method. The activity of cathepsin B (EC 3.4.22.1) and the amounts of total phospholipids, total cholesterol and total proteins were assayed by established procedures.[17–21]

Statistical analyses

Statistical analysis involved three steps: (i) determination of the type of data distribution for each parameter using the Kolmogorov–Smirnow test (Lilliefors) (phospholipid and cholesterol contents were found to be normally distributed whereas cathepsin B activity and drug concentration displayed a log-normal distribution); (ii) analysis of variance (ANOVA) of all data points (or of log thereof) for each parameter examined ($P \leqslant 0.05$ for a significant effect); and (iii) Student's *t*-test analysis of each treated group versus the matching control to determine the threshold in time or extracellular drug concentration at which a given parameter was considered as being significantly influenced by the treatment. All analyses were performed with STATISTICA software (Statsoft Inc., Tulsa, OK, USA).

Results

Computer modelling studies

Figure 1 shows the predicted free azithromycin concentrations in the central compartment for weekly administration of 1200 mg for 3 weeks (panel a, 'prophylactic treatment'), and for daily administration of 500 mg for 14 days, with a follow-up period of an additional 7 days (panel b, 'therapeutic treatment'). At steady-state, the peak and trough values were 0.420 mg/L and 0.002 mg/L respectively for the prophylactic treatment, and 0.255

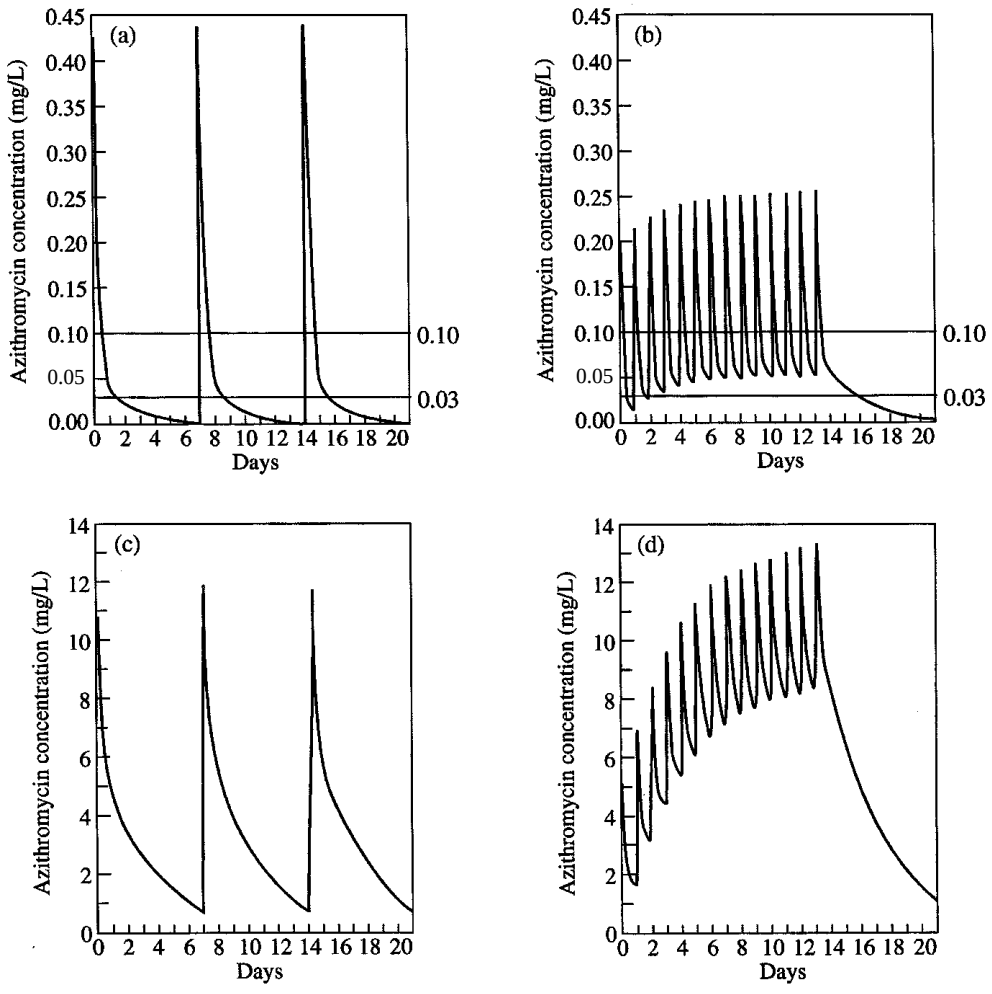

Figure 1. Computer simulation of the free plasma concentration (a and b) and cellular drug content (c and d) with two azithromycin regimens: a weekly dose of 1.2 g for 3 weeks (a and c), and a daily dose of 500 mg for 14 days, with a follow-up period of 7 days (b and d). The horizontal lines in panels a and b indicate extracellular concentrations of azithromycin (0.03 and 0.1 mg/L) used as the two lowest extracellular concentrations in the in-vitro experiments

mg/L and 0.056 mg/L respectively for the therapeutic treatment. Figure 1 also shows the predicted corresponding cellular concentrations, based on an accumulation ratio of 96 (sum of R_1 and R_2 parameters, see Appendix). For the prophylactic treatment (panel c), these would vary between about 12 mg/L (peak) and 0.8 mg/L (trough), with practically no increase in these values from the first administration to subsequent ones. In contrast, the therapeutic regimen would cause a marked rise (approximately four-fold) in the peak and trough cellular drug concentrations, reaching values of about 13 mg/L (peak) and 8 (trough) mg/L after 10 days.

Experimental studies

General design. We used data from the computer-simulated pharmacokinetic studies to set a minimal extracellular concentration at 0.03 mg/L, and established the concentration range from that value up to 20 mg/L to cover the concentrations usually observed during therapy (0.1 mg/L) as well as those used in our earlier high concentration studies.[7,8]

Azithromycin accumulation in cells. Figure 2 shows the actual antibiotic content of cells as a function of the extracellular concentration at the two incubation times. The statistical analysis revealed that the extracellular concentration, the duration of incubation, and the combination of these two parameters all had a significant effect. In the 0.03–1 mg/L range, the cellular content of azithromycin was directly proportional to the extracellular concentration with an accumulation value of 90–140, which is close to our previous observations,[5] and to the value used for the simulation study (96; see above). Yet, the cell:medium drug concentration ratio rose considerably when the drug extracellular concentration was increased to $\geqslant$5 mg/L, reaching ratios of 395–561 at day 8 and 737–957 at day 16 (extreme values), which is considerably

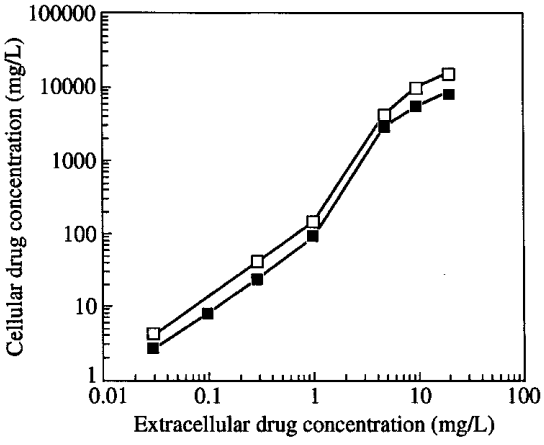

Figure 2. Cellular content of azithromycin in fibroblasts incubated with increasing drug concentrations for 7–8 days (■) or 14–16 days (□). Data, calculated from the drug content of cells determined by reference to the total cell protein and using a cell volume:cell protein ratio of 5 μL/mg,[16] are shown as mean ± SD of at least three culture dishes.

higher than the values recorded for cells incubated for 1 day with the same extracellular concentrations.[5]

Electron microscopy. The morphology of cells incubated with 0.03–1 mg/L azithromycin was examined by electron microscopy. Only lysosomes and related structures showed consistent pathological changes (Figure 3). Compared with controls (panel a), lysosomes accumulated electron-dense, concentric structures (panel b), which showed a lamellar aspect at higher magnification (panels e and f). This lamellar material was easily detectable in cells incubated in 0.03 mg/L azithromycin, even though a fair proportion of lysosomes were still normal. Alterations became prominent in cells incubated in 0.3 or 1 mg/L azithromycin, both with respect to the proportion of lysosomes affected and to the amount of material stored. Detailed observations on partially affected lysosomes suggested that the lamellar structures were built from the periphery of the lysosomes and accumulated inwards in a concentric fashion (panels f, e and d). The size of the

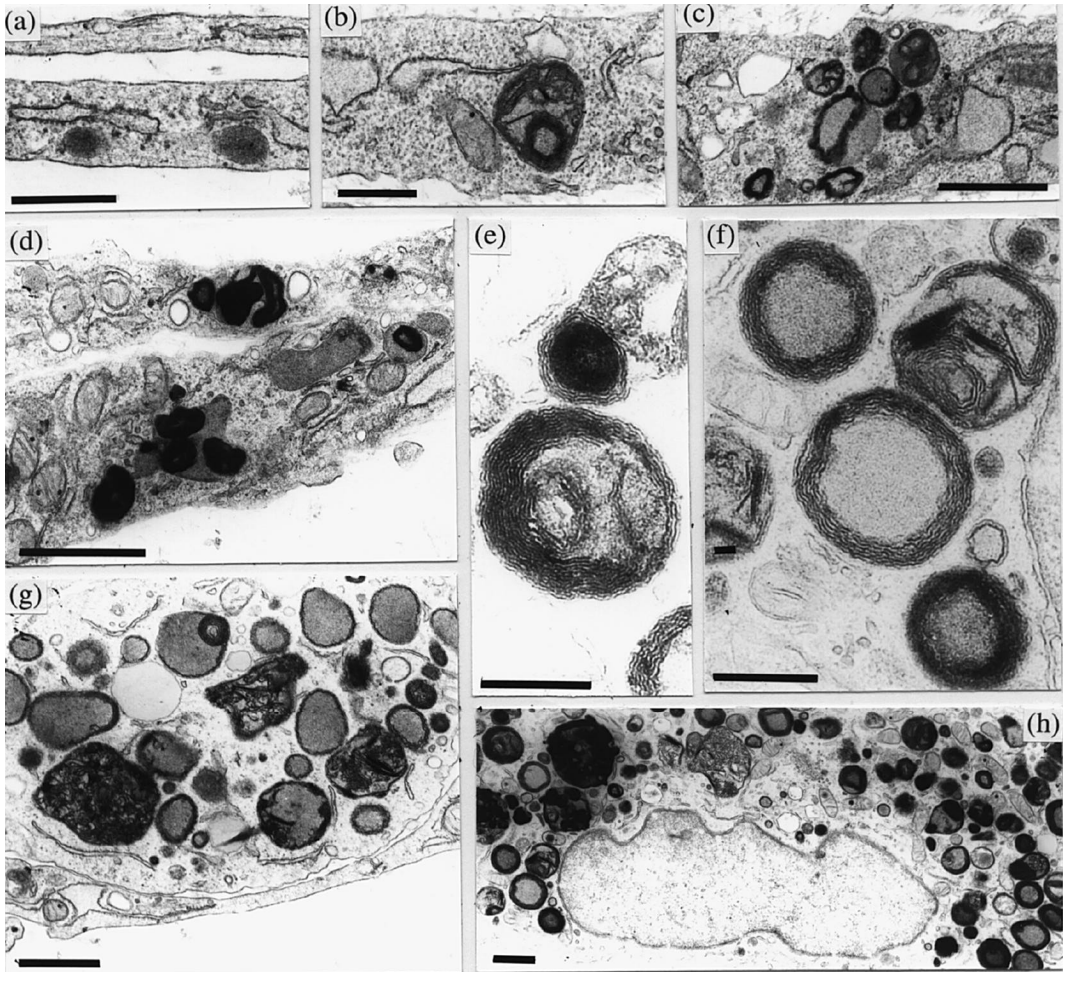

Figure 3. Morphology of lysosomes in control (a) and azithromycin-treated fibroblasts. Panels b–d, g and h illustrate the increasing severity of the lesions with increasing incubation time and/or azithromycin concentrations (panels b and c, 0.03 mg/L for 7 days; panel d, 0.3 mg/L for 14 days; panel g, 0.1 mg/L for 14 days; panel h, 1 mg/L for 7 days). Panels e and f (cells incubated with 1 mg/L azithromycin for 14 days) illustrate the lamellar appearence of structures in lysosomes (e) and its peripheral disposition in partly affected lysosomes (f). Bars represent 0.5 μm (panels a, b, e and f) or 1 μm (panels c, d, g and h).

lysosomes was also enlarged, but not systematically in proportion to the amount of lamellar material stored. A considerable variation was observed within individual cells (panel g) and between cells (panel h shows an exceptionally altered cell).

Biochemical studies. Figure 4 shows the changes in the activity of the lysosomal cysteine-proteinase cathepsin B, and in the contents of total phospholipids and cholesterol. The global statistical analysis showed a significant influence of the extracellular azithromycin concentration on all parameters, as well as an influence of the duration of incubation on cathepsin B activity. Generally, however, significant differences ($P < 0.05$) were observed only with azithromycin concentrations of ⩾1 mg/L.

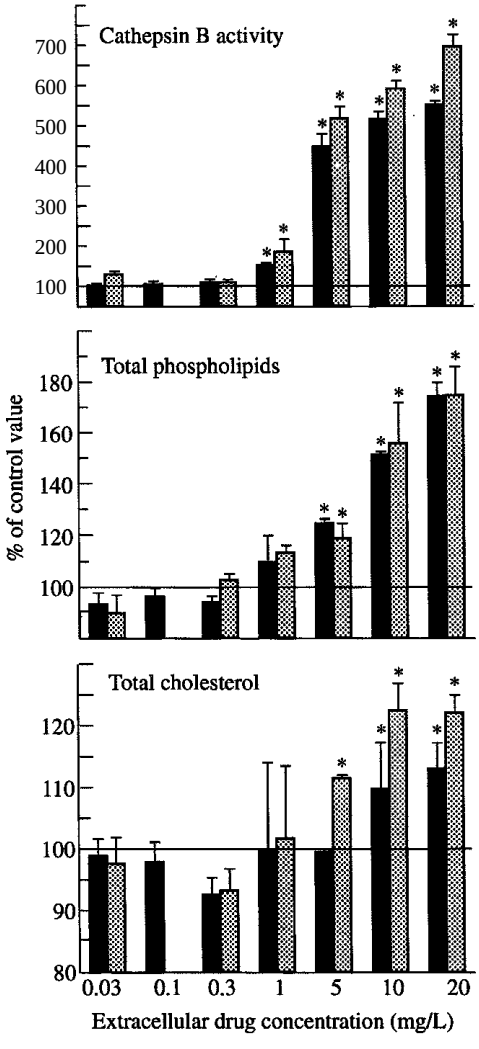

Figure 4. Cathepsin B activity, total phospholipid and total cholesterol contents in fibroblasts incubated with increasing concentrations of azithromycin (■, 7–8 days; ▨, 14–16 days), expressed as percent of control values observed in the corresponding control cells (set at 100% and shown by the horizontal bar). Data are shown as mean ± SD of at least three culture dishes, with the asterisk denoting data significantly different from the control ($P < 0.05$).

Discussion

This study shows that azithromycin induces morphological alterations in the lysosomes of fibroblasts cultured with extracellular concentrations as low as 0.03 mg/L for only 7 days. The alterations are similar to those observed at higher concentrations in the same model,[9] and consist of the deposition of lamellar osmiophilic material, strongly suggesting the accumulation of polar lipids and the development of a lysosomal overloading syndrome. Pertinent biochemical changes become detectable only at ⩾1 mg/L, but this merely indicates that measurements made on *total* cell extracts are relatively insensitive for detecting compositional changes occuring in small organelles, unless the changes are overwhelming. Phospholipids probably accumulate by inhibition of membrane breakdown,[7,22] while cholesterol accumulation probably results from the processing of low-density lipoproteins.[22] Hyperactivity of cathepsin B is probably a secondary effect to lysosomal storage disorder ('thesaurismosis') induced by azithromycin, since it is also observed in other cases of lysosomal overloading by undigested substances.[23]

An intriguing observation is that long-term exposure of cells to ⩾5 mg/L azithromycin leads to much greater accumulation than observed at lower doses or in short-term experiments.[4,7] Since the upsurge in drug accumulation occurs in parallel with the development of a detectable phospholipidosis, it is tempting to speculate that phospholipids participate in the binding of the drug. Cells incubated for 7 days in 10 mg/L azithromycin show a phospholipid excess of approximately 125 nmol/mg protein, representing approximately 30 nEq of free acid charges, which is about half the amount of positive charges brought into the cells by azithromycin (26 μg, i.e. 66 nEq per mg protein). This binding would occur in addition to the previously described proton-trapping of macrolides and other weak organic bases[24,25] which is primarily dependent on the lysosome–extracellular medium pH gradient and is independent of the drug concentration.[25]

The toxicological implications of our findings need to be examined in the light of the actual as well as the reasonably foreseeable uses of azithromycin in humans. The modelling studies strongly suggest that the cellular drug concentrations achievable in human cells are of the same order of magnitude as those observed in rat fibroblasts and that a daily treatment at 500 mg for ⩾8 days may result in extracellular free drug concentrations that will reach and even stay above those causing definite morphological changes. Thus, patients receiving these treatments could experience lysosomal alterations, which might be confirmed by further studies with human biopsies. Extrapolation of the present data to the in-vivo situation needs, however, to be done with caution since cells other than fibroblasts may be less or more sensitive to the effects of azithromycin, and the dynamics of drug intake and efflux that will accompany a once-a-day administration may

result in different effects. These various points are amenable to experimental testing and clinical evaluation. Our data may also be pertinent to the prospective evaluation of other azalides or other macrolide-derived drugs with higher cellular accumulation.

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Appendix

The mathematical model used to predict the cellular concentrations of azithromycin considers that the drug intake process can be described as a bi-exponential process with an initial, rapid phase of approximately 1 h and a secondary, much slower phase lasting several days. This process can be described by the following equation:

$$C_c/C_e = R_1.(1 - e^{-\alpha_1 t}) + R_2.(1 - e^{-\alpha_2 t}) \quad (1)$$

where R_1 and R_2 are dimensionless, α_1 and α_2 are time constants (in h^{-1}), and t is the time (in hours). Expressing the results as a ratio of the apparent cellular concentration (C_c) to the extracellular concentration (C_e) is made possible by converting the measured intracellular concentrations (in milligrams of drug per gram of cellular protein) to mg/L using a conversion factor of 5 μL of cellular volume per milligram of cellular protein as determined earlier for fibroblasts.[16]

Since the intake of drug is associated with its efflux, a compartmental model reflecting Equation 1 can be represented by two cellular compartments, with an input function k_{01} from the extracellular space into the first compartment and rate transfer constants k_{10} in the reverse direction, and k_{12} and k_{21} between compartments 1 and 2. The corresponding differential equations are:

$$dA_1/dt = k_{01} - (k_{10} + k_{12})A_1 + k_{21}A_2 \quad (2a)$$

and

$$dA_2/dt = k_{12}A_1 - k_{21}A_2 \quad (2b)$$

respectively, with

$$C_c/C_e = A_1 + A_2 \quad (2c)$$

Solving this system allows determination of the values of the parameters k_{01}, k_{10}, k_{12}, and k_{21} calculated from R_1, R_2, α_1 and α_2. It is then possible to simulate numerically the apparent drug concentration in fibroblasts exposed to an extracellular concentration of azithromycin, varying as would its free plasma concentration under the conditions of the prophylactic and therapeutic treatments defined above. This is done using the following set of equations:

$$dQ_1/dt = k_{01}C_e(t) - (k_{10} + k_{12})Q_1 + k_{21}Q_2 \quad (3)$$

$$dQ_2/dt = k_{12}Q_1 - k_{21}Q_2 \quad (4)$$

with

$$C_c = Q_1 + Q_2 \quad (5)$$

The Q_1 and Q_2 components are not given here because there is no definite evidence that the two compartments in the model are superimposable on two distinct, physical cellular compartments, even though we know from our previous studies[5] that about two-thirds of azithromycin localizes in lysosomes and the remainder in the cytosol. All data shown refer therefore to the *apparent* total cell concentration, i.e. the amount of drug found in cells per millilitre of cell volume. The data of Carlier *et al.*,[5] and the measurements made in the present study at low doses (0.03 and 1 mg/L) yield values of 23.9, 72.1, 5.814 h^{-1} and 0.01919 h^{-1} for R_1, R_2, α_1 and α_2, respectively. The corresponding values of k_{01}, k_{10}, k_{12} and k_{21} were 140.6 h^{-1}, 5.757 h^{-1}, 0.05682 h^{-1} and 0.01938 h^{-1}, respectively.